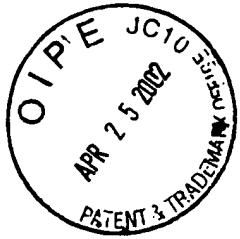


# **APPENDIX A**

**(CLEAN VERSION OF SUBSTITUTE SPECIFICATION EXCLUDING CLAIMS)**

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PATENT

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APPLICATION FOR LETTERS PATENT

for

ALLERGEN DETECTION

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**TITLE OF THE INVENTION:**

**ALLERGEN DETECTION**

**TECHNICAL FIELD**

**[0001]** The present invention relates to allergen detection and, more particularly, to a method and apparatus for indicating allergen levels in dust samples.

**BACKGROUND ART**

**[0002]** It is estimated that up to 80% of the dust particles illuminated by incident sunlight and made visible to the naked eye in a domestic environment are derived from skin. In a warm environment, dust mites feed on skin-derived dust particles, breaking it down by using proteases in their digestive system. Such proteases are found in not insignificant levels in dust mite faeces, and it is now established that it is excreted proteases which act as allergens to individuals who are liable to have an allergic response to house dust. Concentrations of excreted protease are found in relatively high levels in carpets, bedding, pillows and mattresses, all of which provide a suitable environment for dust mites to thrive.

**[0003]** Dust mites are not the only source of proteases found in house dust. For example, proteases from cockroaches are also a source of allergens. Furthermore, it is possible that proteases from cat saliva become airborne as the saliva dries, for example, on the cat's fur. It is likely that such proteases also act as allergens to individuals who are allergic to house dust.

**[0004]** It is known to test house dust in order to determine quantitatively levels of the house dust mite allergen. According to one patent, US 4,806,490, a dust sample is suspended in an aqueous-alcoholic alkali metal hydroxide solution to dissolve or leach out aromatic compounds such as guanine excreted by dust mites, and the resulting solution is mixed with an aromatic diazo compound. A reaction between the aromatic diazo compound and certain excreted aromatic compounds in the solution produces a colour change, with the intensity of the new colour being indicative of the level of excreted proteases in the house dust.

**BRIEF SUMMARY OF THE INVENTION**

**[0005]** According to a first aspect of the present invention, there is provided a method of determining allergen activity in dust, comprising: providing a dust sample; extracting from the

dust sample at least one breakdown component of proteins or peptides; reacting the extracted at least one breakdown component with a colorimetric amine detection reagent; and determining or quantitatively measuring the intensity of any resulting coloration, the allergen activity being proportioned to the intensity of coloration.

**[0006]** The present applicant has appreciated that, in addition to proteases, dust mites excrete the by-products of skin breakdown, including amine compounds, amino acids and relatively small chain peptides, e.g., glycylglycine. In part, the present invention is directed to detecting some of the more abundant, and in some cases chemically less complex, by-products to give an indication of the allergen concentration, rather than targeting one specific compound (e.g., guanine) or type of compounds (e.g., aromatic compounds). This will enable individuals to test particular environments, e.g., individual rooms in a domestic situation to establish that environment's propensity for inducing an allergic response.

**[0007]** The method may further comprise exposing the dust sample to a protease substrate, the protease substrate having immobilised thereon proteins or peptides on which protease in the dust sample may act. The protease substrate may comprise a physical support, such as a matrix or membrane. Thus, in this way, the breakdown components of proteins or peptides will at least in part be generated *in situ*. This may be useful for increasing the concentration of such components, and hence improving subsequent quantitative coloration intensity measurements. If this technique is employed, the exposure time of the dust sample to the protease substrate may need to be controlled (e.g., set at 15 minutes). It is to be noted that, in such a process, the allergen is effectively being measured directly.

**[0008]** The method may further comprise adding a protease inhibitor to the dust sample to suppress activity of a specific protease prior to exposure to the protease substrate. In certain circumstances, it may be necessary to distinguish between dust mite protease and another protease (e.g., from cockroaches), since an individual may be more allergic to one than the other. Differentiation between the types of proteases present in the dust sample can be achieved by differential inhibition of certain specific proteases which may be present. For example, serine protease inhibitors may be used to inhibit specifically serine proteases. The serine protease inhibitors may be selected from the group consisting of organophosphates (e.g., diisopropylphosphofluoridate), sulphonyl fluorides (e.g. phenylmethylsulphonyl fluoride), coumarins (e.g., 3,4-dichloroisocoumarin) and peptide/protein inhibitors (e.g., peptide boronic

acids and aprotinin, respectively) . The use of serine protease inhibitors would allow dust mite allergens (e.g., cysteine proteases) to be detected more readily. On the other hand, cysteine protease inhibitors may be used if dust mite allergens were to be excluded from the test. The cysteine protease inhibitors may be selected from the group consisting of peptide diazomethanes (e.g., z-PheAla-CHN2), peptide epoxides (e.g., E-64 and its derivatives), and cystatins.

**[0009]** In one embodiment of the method, the protease substrate is protease specific, with only a specific protease being able to act on the protein or peptide immobilised on the substrate. In this way, the protease substrate may be chosen to target a specific protease which may be present in the dust sample. If the protease is present in the sample, the specific proteins or peptides immobilised on the substrate will be broken down for subsequent detection. On the other hand, if the specific protease is absent, the proteins or peptides will remain intact and immobilised on the substrate.

**[0010]** The protease substrate may comprise a filter to facilitate extraction of mobile breakdown components of the proteins or peptides immobilised on the protease substrate. The filter may even act as a barrier to the passage of proteases therethrough. The breakdown components extracted from the dust sample may include amines, amino acids or peptides either from the dust sample or from the protease substrate.

**[0011]** The colorimetric amine detection reagent may be 2,4,6-trinitrobenzene sulphonic acid (hereinafter TNBSA).

**[0012]** The at least one breakdown component may be extracted by bringing the dust sample into contact with a surface active agent (surfactant) . Any dust sample solid residues may be separated from the surfactant prior to reacting with the colorimetric amine detection reagent. The surfactant may be an aqueous solution comprising sodium dodecyl sulphate, possibly present in an amount of about~ 5 wt%. The aqueous solution may be alkaline and may also comprise sodium hydrogen carbonate. The dust sample solid residues may be separated by filtration. Removing the solid residues facilitates accurate determination of the intensity of any coloration by reducing the amount of opaque material in the solution.

**[0013]** The intensity of any resulting coloration may be quantitatively determined by comparison with at least one reference colour. The comparison may be with a plurality of different colour references, each selected from the spectrum of colours or range of colour hues attainable. The different colour references may be selected to indicate at least three different

kinds of allergen activity, perhaps corresponding to a macroscopic gradation such as low, medium and high activity.

[0014] The reaction mixture may be preserved by using a stopping agent, e.g., hydrochloric acid, after a preselected incubation or dwell time, e.g., about 2 minutes.

[0015] In order to give reproducible results, the dust sample may be of a predetermined size, e.g., by weight or by volume. The dust sample may be collected by a suction device, perhaps over a predetermined area or time. Variations in the dust sample size may be tolerated since the method represents a gross contamination test, so exact measurements of the dust samples are not necessarily essential.

[0016] In accordance with a second aspect of the invention, there is provided a method of determining allergen activity in dust, comprising: providing a dust sample; providing a protease substrate, the protease substrate having immobilised thereon proteins or peptides labelled with a chromogenic substance; exposing the protease substrate to the dust sample under conditions whereby any protease in the dust sample may act on the immobilised protein or peptide to produce mobile breakdown components labelled with the chromogenic substance; and quantitatively measuring the intensity of any resulting coloration, the allergen activity being proportional to the intensity of the coloration.

[0017] The method may further comprise adding a protease inhibitor to the dust sample to suppress activity of a specific protease prior to exposure to the protease substrate. As before, this will enable the specific protease to be excluded from becoming actively involved in the test, allowing other protease — perhaps present in lower concentrations — to be evaluated. For example, the inhibitor may be a cysteine protease inhibitor if protease allergens other than those from dust mites are to be evaluated.

[0018] In another embodiment of the invention, the protease substrate may be protease specific, with only a specific protease being able to act on the proteins or peptides immobilised on the substrate. In this way, the test may be tailored to evaluate a specific protease, regardless of whether different kinds of protease are present in the dust sample. For example, synthetic substrates with 4—nitroaniline and 2-naphthylamine (chromophores) can be used to distinguish between metaloproteases and aspartic proteases on the one hand (e.g., from cockroaches) and serine and cysteine proteases on the other hand (e.g., from dust mites).

**[0019]** The protease substrate may comprise a filter to facilitate extraction of mobile breakdown components labelled with the chromogenic substance. The filter may act as a barrier to all molecules which are larger than mobile breakdown components labelled with the chromogenic substance.

**[0020]** An example of a protein labelled with a chromogenic substance is azo-albumin. When reacted with a suitable protease, an azo-dye is released.

**[0021]** In accordance with a third aspect of the present invention, there is provided a method of determining allergen activity in dust, comprising: providing a dust sample; extracting from the dust sample at least one component selected from the group consisting of aliphatic amines and aliphatic amino acids; determining the relative concentration of the extracted at least one component; and providing an indication of allergen activity in dependence upon the relative concentration determined.

**[0022]** The relative concentration may be determined by employing a colour indicator sensitive to aliphatic amines and amino acids. The colour indicator may comprise TNBSA.

**[0023]** Any by-products of skin breakdown, particularly aliphatic amines and aliphatic amino acids, present in the dust sample may be linked to dust mite activity. The higher the levels of the by-products in the dust sample, the higher the dust mite activity may be assumed to be. High levels of dust mite activity will produce a correspondingly high amount of protease - the allergens which are largely responsible for providing the allergic reaction to house dust in certain individuals.

**[0024]** In accordance with a fourth aspect of the invention, there is provided a method of determining allergen-generation propensity in dust, comprising: providing a dust sample; exposing the dust sample to a protease able to break down proteins or peptides in human skin cells; reacting the exposed dust sample with a colorimetric amine detection reagent; and quantitatively measuring the intensity of any resulting coloration, the allergen-generation propensity being proportioned to the intensity of the coloration.

**[0025]** An individual may want to evaluate a dust sample to see whether it might support a high level of dust mite activity, even before the allergen levels have built up to significant, detectable levels. If the dust sample contains relatively high levels of human skin cells, the protease supplied will produce breakdown components which will react with the reagent and thereby be detected by colour evaluation. By containing relatively high levels of

human skin cells, the dust sampled could in theory support high concentrations of dust mites. Such information may be a useful warning to those individuals who are allergic to dust mite protease.

**[0026]** The colorimetric amine detection reagent may be 2,4,6-trinitrobenzene sulphonic acid. The intensity of any resulting coloration may be quantitatively measured by comparison with at least one reference colour.

**[0027]** In accordance with another aspect of the present invention, there is provided apparatus for use in a domestic environment for determining indicating allergen levels. The apparatus may comprise a kit comprising a first chamber comprising a surfactant for extracting from a dust sample at least one breakdown component of proteins and peptides; a second chamber comprising a colorimetric amine detection reagent; means for quantitatively measuring the intensity of any coloration resulting from reacting the extract-containing surfactant and the colorimetric amine detection reagent; and means for indicating relative levels of allergen activity in the dust sample based on the quantitative measurement.

**[0028]** The apparatus may further comprise a filter for filtering dust sample solid residues from the surfactant before reacting with the colorimetric amine detection reagent, which may be TNBSA. One of the two chambers may have the capacity to receive the contents of the other chamber. Preferably, the second chamber has the capacity to hold the colorimetric amine detection apparatus and the surfactant.

**[0029]** The quantitative measuring means may comprise at least one colour reference, against which the colour of the solution may be compared. The indicating means may comprise a scale, e.g., low, medium and high activity, which is linked to the intensity of any coloration measured. For example, if the colour of the solution is determined by eye as being about the same as the colour reference, this could correspond to medium allergen activity. Divergence either side of the colour reference would then correspond to low or high activity as appropriate.

**[0030]** The apparatus may further comprise a third chamber comprising a stopping reagent to limit the reaction between the extract-containing surfactant and colorimetric amine detection reagent, e.g., TNBSA.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0031] An embodiment of the invention will now be described with reference to the accompanying drawings, in which:

[0032] Figure 1 shows schematically apparatus for determining dust mite activity in accordance with the present invention;

[0033] Figure 2 shows schematically the use of apparatus shown in Figure 1;

[0034] Figure 3 is a flow chart illustrating one method of determining allergen levels according to the invention; and

[0035] Figure 4 is a flow chart illustrating another method embodying the invention.

## DETAILED DESCRIPTION OF THE INVENTION

[0036] The apparatus 10 of figure 1 comprises three parts: an upper part 12 which contains in a first chamber 14 0.10 litres of a 0.1M solution of sodium hydrogen carbonate containing 5 wt% of sodium dodecyl sulphate; a middle part 16 which is a snug but sliding fit in both the upper part 12 and the remaining part; and a lower part 18 which contains a tablet of TNBSA and a stopping reagent of 1.0M hydrochloric acid. The solution in the first chamber 14 is sealed in the upper part 12 by a frangible seal 20. The middle part 16 comprises a filter 22 above which is provided a cup 24 for receiving a dust sample. The middle part 16 has a leading profile 26 which is pointed to facilitate breaking the frangible seal 20. A second chamber 27 is formed by the middle and lower parts. The lower part 18 includes a frangible seal 28 disposed between the tablet of TNBSA and the stopping reagent which is sealed in a third chamber 29.

[0037] The use of the apparatus 10 is now described in stages with reference to figure 2:

Stage 1 A sample of dust of predetermined size is placed in cup 24.

Stage 2 The middle part 16 is inserted into the upper part 12, such that the profile 26 ruptures the seal 20.

Stage 3 The solution in the first chamber comes into contact with the dust sample. Any chemicals including amines, amino acids and peptides present in the dust sample are extracted and pass through filter 22 and into the second chamber 27 where they come into contact with the tablet of TNBSA.

Stage 4 After about 2 minutes, the middle part 16 is pushed far enough into the lower part 18 to rupture seal 28, enabling the stopping reagent in the third chamber 29 to prevent further reaction. The colour of the resulting solution is compared with a colour key which is calibrated to give an indication of the level (e.g., low, medium or high) of dust mite activity in the dust sample.

Example

[0038] A dust sample was collected from an old mattress (where dust mite activity may be expected to be high), and a blank sample and test samples of GlycylGlycine in varying concentrations (20-200 micrograms) were used as controls. The dust, blank and test samples were washed with 0.1M NaHCO<sub>3</sub> 0.5M NaCl (ph 8.3) and then tested with ThBSA of various concentrations e.g., diluted to 1 part in 10, 1 part in 50 and 1 part in 100. It was found that a dilution of 2. part in 50 was the optimum dilution for sensitivity and blank color. Using such a dilution, the experiment yielded visual results for both the dust and all test samples, but not the blank sample. The visual results could then be assessed and compared to give an indication of dust mite activity in the old mattress.

[0039] The method used in the example may be summarised and developed with reference to Figure 3. A dust sample provided at step 50, possibly by using a suction device to collect dust from furniture or carpets. A protease inhibitor (e.g., serine protease inhibitor) is added at step 52) to enable a particular protease (e.g., cysteine protease) to be targeted. Next, at step 54, the dust sample is exposed to a protease substrate which is susceptible to the proteases present. Protein or peptide breakdown components from the dust sample or protease substrate are then extracted at 56 and are reacted at 58 with the calorimetric amine detection reagent (TNBSA). The presence of free amino groups causes an orange-coloured product, the intensity of which is measured at 60 to give an indication of allergen levels.

[0040] Instead of using a protease inhibitor (step 52), the protease substrate may be selected to be protease specific. In other words, the protease substrate may contain proteins or peptides which require the presence of specific proteases under evaluation before yielding detectable breakdown components.

[0041] An alternative method is illustrated in Figure 4, and again starts with the provision of a dust sample (again step 50). A protease-specific substrate is provided at 70, the

substrate having immobilised thereon proteins or peptides which require specific proteases before yielding breakdown components. The immobilised proteins or peptides are also labelled with a chromogenic substance. At step 72, the substrate is exposed to the dust sample. The presence of the specific proteases in the sample will break down the immobilised proteins or peptides, releasing the chromogenic substance, causing coloration of the solution. The intensity of the coloration is measured at 74 to give an indication of allergen levels.

**[0042]** Instead of using a protease-specific substrate (step 70), the protease substrate may be nonspecific, but still labelled with the chromogenic substance. If a specific protease is still to be targeted, this may be accomplished using protease inhibitors (step 52 of Figure 3).